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Transcriptional Regulation of DUOX-Dependent Reactive Oxygen Species Production Against Bacterial Infection in the Gut of Cat Fleas (Ctenocephalides felis).

Ryne W. Maness

TRANSCRIPTIONAL REGULATION OF DUOX-DEPENDENT REACTIVE OXYGEN SPECIES PRODUCTION AGAINST BACTERIAL INFECTION IN THE GUT OF CAT FLEAS (*CTENOCEPHALIDES FELIS*).

by

RYNE MANESS

(Under the direction of Lisa D. Brown)

ABSTRACT

Fleas (Order Siphonaptera) are opportunistic blood feeders that parasitize a wide variety of mammals and birds. They also transmit bacterial pathogens that cause diseases in humans (e.g., murine typhus, flea-borne spotted fever, cat scratch disease, and plague). Because they acquire infectious pathogens while blood feeding, the flea gut is considered to be the initial site of infection. While immune responses have been well documented in other disease vectors, few studies have identified the immune mechanisms involved in defense of the flea gut. In other hematophagous insects, the synthesis of reactive oxygen species (ROS) is the immediate immune defense mechanism against foreign microbes. To investigate the role of ROS in flea gut immunity, cat fleas (*Ctenocephalides felis*) were orally infected with the model insect pathogen *Serratia marcescens*. Specifically, fleas were treated with an antioxidant to reduce the amount of microbicidal ROS before infection, and then *S. marcescens* infection loads were measured. Additionally, we measured hydrogen peroxide (ROS) levels, and the relative quantity of mRNA for select genes associated with DUOX, a surface protein of epithelial cells responsible for ROS production. Four experimental groups were examined: (1) *S. marcescens*-infected fleas; (2) fleas fed an antioxidant; (3) fleas fed an antioxidant and then infected with *S. marcescens*, and (4) fleas fed on untreated blood (control). Overall, these results show that ROS levels in the gut increase in response to infection, and the signaling pathway for DUOX activation is tightly regulated. This study provides evidence that ROS is a key mechanism for early gut defense in cat fleas.

INDEX WORDS: Fleas, Cat flea, *Ctenocephalides felis*, Reactive oxygen species, ROS, DUOX

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DEDICATION

For MK, this would not be possible without your love and support. Your patience and kindness truly inspire me every day.

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CHAPTER 1

INTRODUCTION

Purpose of Study

Fleas (Order Siphonaptera) are obligate hematophagous insects that survive as external parasites of birds and mammals, including humans. Those species that feed on people, as well as their companion animals, often have medical and veterinary importance (Durden and Hinkle 2019). For example, the cat flea (*Ctenocephalides felis*) is a common household flea pest responsible for flea allergy dermatitis, cases of severe anemia (especially in puppies and kittens), and transmission of a wide variety of infectious disease agents (Azad *et al.* 1997, Dobler and Pfeffer 2011, Rust 2017). In particular, cat fleas serve as vectors of bacterial pathogens that cause acute human diseases, such as murine typhus (caused by *Rickettsia typhi*), cat scratch disease (*Bartonella henselae*), and flea-borne spotted fever (*Rickettsia felis*) (Eisen and Gage 2012). Of these pathogens, *R. typhi* and *B. henselae* are considered ongoing threats to public health because of their high prevalence among wild-caught fleas and the possible medical complications that arise from human infections (Bitam *et al.* 2010, Van der Vaart *et al*. 2014, Mullins *et al*. 2018, Abdullah *et al*. 2019, Blanton *et al*. 2019, Breitschwerdt *et al*. 2019).

The cat flea has a cosmopolitan distribution and will feed on almost any available mammalian host (Rust 2017). These opportunistic blood-feeders can acquire infectious agents while feeding on an infected host, and later transfer the pathogen to uninfected hosts during subsequent feeding events (Bitam *et al*. 2010). The two primary modes of pathogen transmission by fleas are orally through regurgitation (anterior station) or via defecation of fecal material (posterior station) (Bitam *et al.* 2010). Both *R. typhi* and *B. henselae* are transmitted through the flea feces, while *R. felis* is primarily transmitted through flea bites, though there is evidence that *R. felis* can be transmitted posteriorly as well (Eisen and Gage 2012, Legendre and Macaluso 2017). Although *C. felis* serves as the main vector of these infectious agents,

these microorganisms are foreign to the flea host. Thus, fleas will mount an innate immune response to combat infection by these pathogens (Brown 2019).

Two of the most characterized immune signaling pathways in insect immunity are the immune deficiency (IMD) pathway for Gram-negative bacteria and the Toll pathway for Gram-positive bacteria (Hoffman and Reichhart 2002). Both pathways have been studied extensively in the fruit fly (*Drosophila melanogaster*) and other insect vectors (e.g., mosquitoes) (Valanne *et al*. 2011, Myllymäki *et al.* 2014, Barletta *et al*. 2017), but relatively little is known about these immune signaling pathways in fleas. A previous study by Rennoll *et al.* (2017) examined activation of the IMD pathway in *C. felis* in response to *R. typhi* infection. Because the cat flea genome was not sequenced at the time of the study, the researchers first analyzed the *C. felis* transcriptome to show that IMD pathway components are conserved between *D. melanogaster* and *C. felis.* Next, the role of the IMD pathway in *C. felis* immunity was explored by silencing select pathway genes (*Relish* and *Imd*) prior to infection with *R. typhi*. As a result, knockdown fleas experienced higher bacterial loads on average compared to wild-type individuals, thus signifying the importance of the IMD pathway in *C. felis* immunity (Rennoll *et al.* 2017). Additionally, IMD has been implicated to activate other immune pathways in *Drosophila* and could potentially play similar roles within cat fleas (Kim and Lee 2014). Despite this knowledge, the study of immune signaling in cat fleas is still in its infancy.

The primary site of infection for cat fleas is the gut, where blood is digested and absorbed throughout its entirety (Reinhardt 1976, Eisen and Gage 2012). In *Drosophila*, the earliest host defense against pathogens in the gut is associated with the epithelial cells lining the gut wall (Ha *et al.* 2005b). These cells produce reactive oxygen species (ROS), such as hydrogen peroxide, through the activation of the membrane-bound enzyme, dual oxidase (DUOX) (Donkó *et al*. 2005). There are two primary pathways to activate DUOX, the Gαq-PLCβ-Ca^{2+} pathway (DUOX activity pathway) and the MEKK1-MEK3-p38-ATF2 pathway (DUOX expression pathway) (Ha *et al.* 2009b; Fig. 1). While both pathways are required for production of ROS, only the DUOX expression pathway is mediated by bacterial

peptidoglycan (Leulier and Royet 2009, Zug and Hammerstein 2015). Peptidoglycan is the primary peptide found on the surface of Gram-positive bacteria, or below the outer membrane in Gram-negative bacteria, and constitutes the majority of the cell wall (Vollmer *et al*. 2008). Because it is highly conserved among bacteria and important for cell function, peptidoglycan is a common pathogen-associated molecular pattern (PAMP) recognized by the IMD signaling pathway, which merges into MEKK1 of the DUOX expression pathway (Wang *et al*. 2019).

After recognition of peptidoglycan, IMD activates MEKK1 through an unknown mechanism to begin phosphorylation of the DUOX expression pathway and basal production of ROS (Leulier and Royet 2009, Chakrabarti *et al*. 2014). In contrast, activation of the DUOX activity pathway is triggered by a non-peptidoglycan ligand, bacterial-derived uracil (Lee e*t al*. 2013). Together, activation of both pathways leads to the stable production of hydrogen peroxide (ROS), which is then converted into HOCl, a microbicidal agent, by the peroxide homology domain (PHD) (Ha *et al.* 2005b). While DUOX-dependent ROS generation limits bacterial survival, the presence of free radicals is also detrimental to the surrounding host cells (Ha *et al*. 2005a, Mikonranta *et al*. 2014). Overall, these molecules are vital to protection of epithelial cells from infection, but must be tightly regulated to avoid damaging the surrounding host tissues (Ha *et al*. 2005a).

Infection-induced ROS generation has been observed in the gut of several species of blood-feeding insects, including sand flies, mosquitoes, and the Oriental rat flea (*Xenopsylla cheopis*) (Diaz-Albiter *et al*. 2012, Kumar *et al*. 2003, Molina-Cruz *et al*. 2008, Oliveira *et al.* 2011, Zhou *et al*. 2012). As such, I hypothesize that ROS plays an important role in the gut immune response of cat fleas. In order to characterize DUOX-dependent ROS generation in the cat flea, hydrogen peroxide (ROS) levels and transcription levels of select DUOX pathway genes were measured after flea infection with a model bacterium (*Serratia marcescens*). These results indicate that ROS levels are elevated in infected fleas, and expression of genes in the DUOX pathway are tightly regulated. Overall, these data suggest that ROS provides early host defense against ingested bacterial pathogens in the gut of cat fleas.

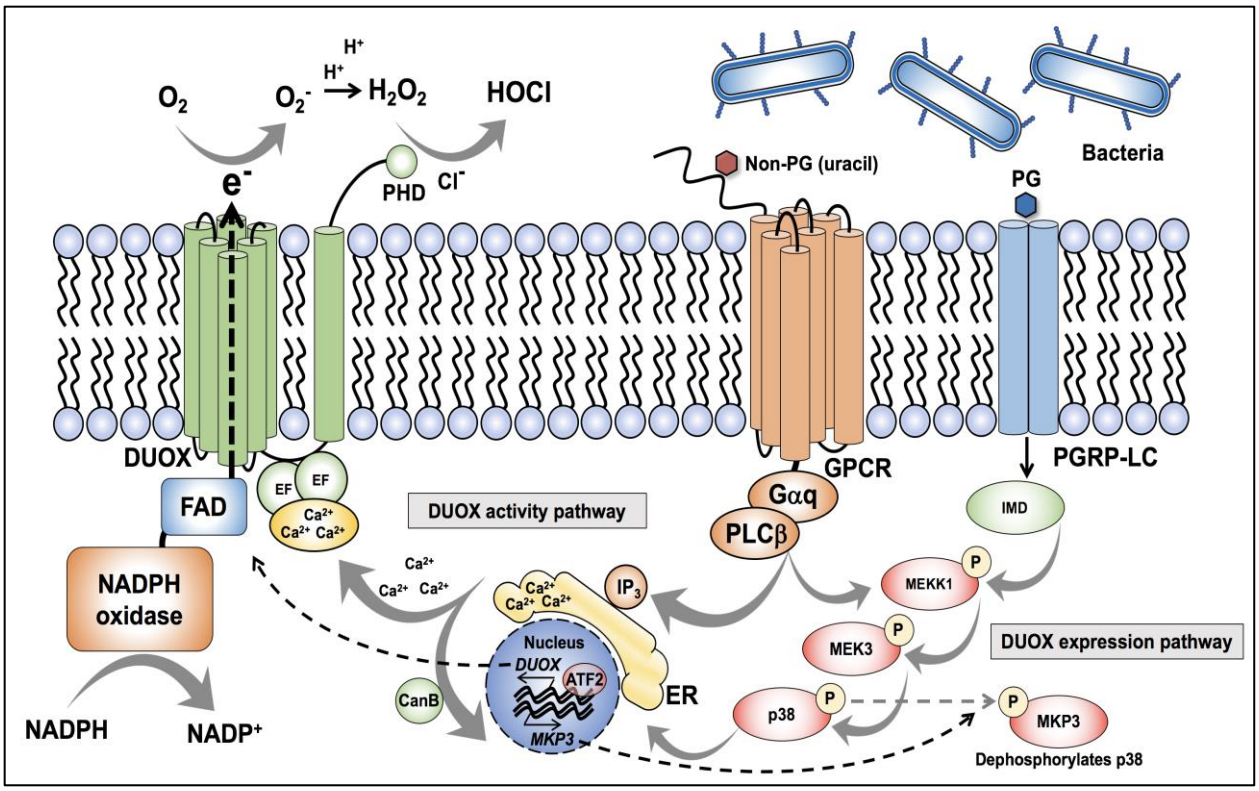

Figure 1. DUOX signaling pathways that regulate ROS production in the gut of fruit flies (*Drosophila melanogaster*). Dual oxidase (DUOX) is a membrane-bound, multi-domain enzyme consisting, in part, of a bound flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate oxidase domain (NADPH) that catalyzes the reduction of molecular oxygen to generate H_2O_2. This is accomplished by using NAPDH as an electron donor. Subsequently, the peroxidase homology domain (PHD) of DUOX converts H_2O_2 into microbicidal HOCl in the presence of chloride (Cl^-). Two signaling pathways regulate the generation of DUOX-dependent H_2O_2 molecules, referred to as the DUOX activity pathway and the DUOX expression pathway. In the DUOX activity pathway, uracil activates the G protein alpha subunit q (Gαq). The activation of Gαq initiates phospholipase Cβ (PLCβ)-dependent production of inositol-1,4,5-triphosphate (IP_3). This triggers mobilization of intracellular calcium ions (Ca^{2+}) out of the endoplasmic reticulum (ER), which after binding with an intracellular EF hand domain (EF), increases DUOX enzymatic activity. Transcription of *Duox* is then upregulated via p38-mediated activation of ATF2. Expression of p38 is controlled by a peptidoglycan (PG)-independent PLCβ pathway and a PG-dependent IMD pathway that merge into MEKK1. Recognition of PG by a peptidoglycan recognition protein LC (PGRP-LC) is believed to then activate the MEKK1, MEK3, and p38 kinase cascade. Under normal host conditions, p38 is inactivated in order to protect host cells from damage caused by the production of free radicals. This inhibition is controlled by the release of calcineurin B (CanB), which induces activation of MKP3, the protein responsible for the dephosphorylation of p38 and subsequent reduction in *Duox* transcription.

CHAPTER 2

METHODS

Flea rearing and maintenance

Newly emerged, unfed, adult cat fleas (*C. felis*) were purchased from Elward II Laboratory (Soquel, CA, USA). Adult fleas were maintained on bovine blood (HemoStat Laboratories, Dixon, CA, USA) within an “artificial dog" feeding system (Wade and Georgi 1988). This system was kept in an environmental chamber at 25°C and 55% relative humidity (RH). Fleas were then stored in cages covered by a thin mesh film to allow for access to bloodmeals. Next, bloodmeals were aliquoted into containers surrounded with Parafilm® and heated to 37°C, the relative body temperature of a potential flea host, using a heat lamp while placed on top of the flea cages. Following feeding and successful mating within the chamber, eggs were collected onto sand (QUIKRETE®) in a Petri dish to complete development to adulthood, and hatched larvae were fed the dried feces from blood-feeding adults. Immature stages were reared at 25°C and ≥ 85% RH in an incubator with no light source and organized into containers for future use. To optimize adult survival rates, both sexes were stored together during experimental treatments, but only female fleas were processed for further use in the study.

Creation of infectious bloodmeal and measurement of internal bacterial load in fleas

Serratia marcescens cultures (Carolina Biological Supply Company, Burlington, NC, USA) were grown overnight in a shaking incubator at 25°C in Difco™ nutrient broth (Sparks, MD, USA). Infection dose was estimated prior to beginning an experiment by measuring the optical density (OD_{600}) of the bacterial culture in a Biophotometer D30 (Eppendorf AG, Hamburg, Germany). After obtaining an OD_{600} of approximately 5, a two-fold serial dilution was then plated on Difco™ nutrient agar (NA) (Sparks, MD, USA) to determine the infection dose fed to the fleas. The plated infection dose was then incubated for 48 hours at room temperature (25°C) and resultant colony forming units (CFUs) were counted to provide an estimate of the inoculation dose fed to the fleas. Prior to creation of an infected bloodmeal,

600 µl of bovine blood was heated at 55°C for 10 minutes to inactivate mammalian immune factors. Bacterial cultures were then pelleted by centrifugation at 13,000 g for 5 minutes and re-suspended in the heat-inactivated blood. Fleas were starved for 3-4 hours before feeding on the infected bloodmeal. After feeding, flea guts were dissected, homogenized, serially diluted, plated on NA, and grown for a period of 48 hours at room temperature. The resultant *S. marcescens* CFUs were then counted.

Measurement of flea infection rates during and after exposure to an infectious bloodmeal

To determine a timeline of infection with *S. marcescens*, cat fleas (n = 100) were exposed to either control blood or infected blood in the artificial feeding system. To test infection rates during feeding, a subset of fleas (n = 10) were processed individually while they were actively feeding on a *S. marcescens*-infected bloodmeal at 1, 3, 6, 12, and 24 h. Subsequent bacterial load or clearance of *S. marcescens* was determined in individual fleas (n = 10) at 48, 72, 96, 120, and ≥144 h following removal of the infected bloodmeal at 24 h. Flea guts were dissected, homogenized, serially diluted, and plated on NA as described above to measure bacterial load.

Measurement of ROS efficiency by antioxidant treatment

Antioxidants are naturally produced by organisms as a defense against free radicals (ROS). These molecules are capable of donating electrons to free radicals thereby neutralizing them and protecting the host from ROS damage (Pham-Huy L. *et al*. 2008). To test the importance of ROS, each group of fleas tested was fed an antioxidant before feeding on an *S. marcescens*-infected bloodmeal. Antioxidant groups were fed approximately 20 mM of N-acetyl-L-cysteine for a total of 48 hours before receiving either regular blood or infected-blood. Next, bacterial concentrations were determined using the previously described methods (*i.e.*, dissection, mixing, and plating on NA). A total of five independent trials were conducted with approximately 20 individual fleas.

Quantification of ROS levels by hydrogen peroxide assays

Four groups of fleas were given one of the following treatments: 1) untreated bovine blood (control), 2) *S. marcescens*-infected blood, 3) blood mixed with an antioxidant followed by untreated blood, and 4) antioxidant mixed blood followed by infected blood (Table 1). After successful feeding, the level of hydrogen peroxide (ROS) was measured using the Pierce™ Quantitative Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions for the lipid-compatible procedure. Due to the presence of iron from blood in the flea gut, three samples were created, a sample with methanol only, a sample with Bond-Breaker™ TCEP Solution (Thermo Fisher Scientific, Waltham, MA, USA) and a blank sample. Two replicates for each of the samples were then made and absorbance was measured in a Synergy H1 microplate reader (BIOTEK®, Winooski, VT, USA) set to 595 nm, based on manufacturer's recommendations. The average readings from the blank samples were then subtracted from the methanol and TCEP samples. Finally, the average TCEP absorbance was subtracted from the average methanol-only absorbance at each peroxide concentration to create a standard curve. Each group included approximately 20 individuals and three independent trials were conducted.

Quantification of DUOX-related gene expression by qPCR

Six target genes of interest (GOI) were selected based on their function in the DUOX expression pathway in *D. melanogaster*: *MEKK1*, *MEK3*, *p38a*, *ATF2*, *Duox,* and *MPK3*. Gene sequences were obtained through the use of FlyBase and NCBI databases (Table 2) (Kim *et al*. 2011, Mcintosh *et al*. 2016, Rennoll et al. 2017). Briefly, protein sequences from *D. melanogaster* were compared against the *C. felis* transcriptome (GenBank accession #: GAYP00000000) using tBLASTn. Transcripts that produced the most significant alignment were then used to compare against the *C. felis* genome (GenBank accession #: GCA_003426905.1) using BLASTn (Table 2). Finally, the most significant alignments were examined for potential RNA splice sites, and primer pairs were designed in Primer3 (version 0.4.0) from *C. felis* transcripts such that the amplicon spans a potential intron (Table 3). Candidate reference genes,

Glyceraldehyde 3 phosphate dehydrogenase (GADPH), 60S ribosomal protein L19 (RPL19), and elongation factor-1α (Ef), were created from *D. melanogaster* sequences as previously described, and used as endogenous controls (Table 4) (Mcintosh *et al*. 2016).

Cat fleas (n = 100) were then exposed to either untreated blood (control), *S. marcescens*-infected blood, antioxidant-treated blood, or a combination of antioxidant-treated blood followed by *S. marcescens*-infected blood through an artificial feeding system (Table 5). To monitor the temporal dynamics of DUOX gene expression, infected bloodmeals were removed after a period of four hours or 24 hours. Flea guts were dissected, pooled by treatment (approximately 70 fleas), and homogenized in separate microcentrifuge tubes with 300 uL of Trizol™ reagent (Invitrogen, Carlsbad, CA, USA). To create complementary DNA (cDNA) for gene expression analysis, messenger RNA (mRNA) was extracted and purified using the Direct-Zol RNA Miniprep Plus kit (Zymo Research, Irvine, CA, USA). Concentrations of mRNA were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5 µg of RNA was used as template for cDNA synthesis using the High-Capacity RNA-to-cDNA™ kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Real-time quantitative PCR reactions were pre-mixed in a 35-µl volume containing 2X PowerUp™ SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 µM of each primer, DNase/RNase-free water, and 5 µl of cDNA template (1/16 dilution). Additionally, nuclease-free water was used in place of template as a negative control, and no RT reactions (sterile water was added instead reverse transcriptase) were performed to confirm the absence of genomic DNA. The mixtures were then aliquoted in triplicate 10-µl reactions onto MicroAmp® Fast Optical 96-well reaction plates and run on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Default thermal cycling conditions were used as specified in the master mix user guide.

A standard curve was generated to allow for the comparison of relative gene expression of the GOIs, by normalizing based on the chosen reference genes (GAPDH, RPL19, and Ef) for both untreated controls and the treatment groups. Two-fold serial dilutions of stock cDNA were used to create an eight-

point standard curve for each GOI. The quantity of the GOI was then extrapolated from the standard curve and normalized based on the quantity of reference gene (Normalized GOI = GOI /GeoMean [Refence Genes]). Finally, the relative quantity (RQ) was determined for the treatment groups by taking the normalized GOI from the treatment groups and dividing it by the normalized GOI of interest from the untreated controls (RQ = Normalized GOI [treatment groups] / Normalized GOI [untreated control]).

Statistical analyses

Bacterial loads following antioxidant treatment were compared by an unpaired t-test. Infection intensity, infection prevalence, and hydrogen peroxide levels were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. Additionally, DUOX gene expression data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test to compare relative expression of genes in treatment groups to untreated control fleas. Groups were considered to be significantly different at the p-value ≤ 0.05. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad software).

Table 1. Treatment schedule of experimental bloodmeal groups. Fleas were organized into four groups and orally exposed to one of the following treatments: (1) untreated blood (control); (2) blood infected with *S. marcescens*; (3) blood mixed with an antioxidant, and then untreated blood; or (4) blood mixed with an antioxidant, and then blood infected with *S. marcescens*. Groups were fed over a period of 120 hours with bloodmeals changed at 24 hours, 72 hours, and 120 hours.

Group	24 Hours	72 Hours	120 Hours
1	Untreated Blood	Untreated Blood	Untreated Blood
2	Untreated Blood	Untreated Blood	Infected Blood
3	Antioxidant Blood	Antioxidant Blood	Untreated Blood
4	Antioxidant Blood	Antioxidant Blood	Infected Blood

Table 2. Gene names, FlyBase gene IDs, and NCBI IDs for *Drosophila melanogaster* proteins, *Ctenocephalides felis* transcripts, and *C. felis* genes.

Gene	FlyBase ID	Protein ID	Transcript ID	Genome ID
Duox	FBgn0283531	NP_001259968.1	GAYP02000964.1	NW_020539725.1
p38a	FBgn0015765	NP_732959.1	GAYP02010133.1	NW_020537012.1
MEKK1	FBgn0024329	NP_732373.1	GAYP02016189.1	NW_020539725.1
ATF2	FBgn0265193	NP_001033973.1	GAYP02010810.1	NW_020538040.1
MEK3	FBgn0261524	NP_477162.1	GAYP02010221.1	NW_020539725.1
MKP3	FBgn0036844	NP_001262032.1	GAYP02013936.1	NW_020539724.1

Table 3. Gene function and primers used for quantitative RT-qPCR.

Gene	Function	Forward primer (5'→3')	Reverse primer (5'→3')
Duox	NADPH oxidase activity	CGCATGAGCAATAAGGGTTT	AGAGAATGGCTTCATGCAA
p38a	MAP kinase activity	ATCAGTTCCTGGGAACAATG	TTGGGCTAGCACGACCTACT
MEKK1	MAP kinase activity	CAACAGCAACATCAGCATTG	GATGTCGCTTGCGTGATAGA
ATF2	Transcription factor for Duox protein	TCTGAATTCCGAGTGGTGTG	TCACCAACTGGAACAGGAAA
MEK3	MAP kinase activity	GGGATGTAAAGCCATCAAACA	ACCAAGTGACCAGACATCAGA
MKP3	Inactivation of MAPK activity	CCGCTGTGCAAACTGATAGA	GCTCACCTTTGCCTGGAAG

Table 4. Reference gene function and primers used for quantitative RT-qPCR.

Gene	Function	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	Glycolysis	ACCCAAAAGACTGTGGATGG	CGGAATGACTTTGCCTACAG
RPL19	Structural protein in the ribosome	TACAGCTAATGCCCGTACACC	TTCAACAAACGCCTCAGGAC
Ef	Translation	TCGTACTGGCAAATCCACAG	CATGTCACGGACAACGAAAC

Table 5. Experimental schedule of flea treatment groups for RT-qPCR. Groups of fleas were organized into cages and fed on either untreated blood, antioxidant blood, or *S. marcescens*-infected blood. Flea guts were then extracted via dissection at 24 hours-post infection, with the exception of one infection group which was dissected at four hours-post infection.

Group	24 Hours	72 Hours	120 Hours	124 Hours	144 Hours
Control	Feeding on Untreated Blood	Feeding on Untreated Blood	Feeding on Untreated Blood	Feeding on Untreated Blood	Dissection
Infection (4 Hours)	Feeding on Untreated Blood	Feeding on Untreated Blood	Feeding on Infected Blood	Dissection	**N/A**
Infection (24 Hours)	Feeding on Untreated Blood	Feeding on Untreated Blood	Feeding on Infected Blood	Feeding on Infected Blood	Dissection
Antioxidant	Feeding on Antioxidant Blood	Feeding on Antioxidant Blood	Feeding on Untreated Blood	Feeding on Untreated Blood	Dissection
Antioxidant + Infection	Feeding on Antioxidant Blood	Feeding on Antioxidant Blood	Feeding on Infected Blood	Feeding on Infected Blood	Dissection

CHAPTER 3

RESULTS

Bacterial load in the flea gut increased with exposure time to an infectious bloodmeal

To establish a timeline of *S. marcescens* infection in cat fleas, a subset of fleas (n = 10) were processed at 1, 3, 6, 12, and 24 hours while actively feeding on an infected bloodmeal, and then at 48, 72, 96, and ≥120 hours following removal of the infected bloodmeal at 24 hours. Individual guts were dissected and plated to determine infection intensity (average number of CFUs per *S. marcescens*-infected flea) and infection prevalence (percentage of fleas infected with *S. marcescens*) over the time course described above.

Bacterial loads were relatively stable between 1 and 12-hours of feeding on an infected bloodmeal. Specifically, the average infection intensity was 3,456.00 CFU (± 809.30 SEM) at 1 hour, declined to 2,676.00 CFU (± 1,243.00 SEM) at 3-hours, decreased further at six hours (557.60 CFU ± 175.60 SEM), and increased to 6,440.00 CFU (± 2,796.00 SEM) at 12-hours (Fig. 2). At 24-hours, the quantity of *S. marcescens* colonies nearly eclipsed 30,000 CFU (29426 CFU ± 8089.00 SEM) per infected flea, while all other time points never reached 7,000 colonies per infected flea. Following removal of the infectious bloodmeal, bacterial loads decreased to 752.60 CFU (± 368.80 SEM) per flea at 48 hours and reached a low of 368.70 CFU (± 119.90 SEM) at 96 hours (Fig. 2). Overall, bacterial intensity increased by 751.00% from 1 to 24 hours of feeding on an infected bloodmeal and decreased by 95% from 24 to ≥120 hours following the removal of the infectious bloodmeal (Fig. 2; Tukey's: 1 vs. 24, $p = 0.0133$ and 24 vs. ≥120, $p = 0.0062$.

While feeding on an infected bloodmeal, the percentage of infected fleas was 70% (± 11.55% SEM) at one-hour post exposure, then decreased to 60.00% (± 6.67% SEM) at three-hours and 55.00% (± 6.67% SEM) at six-hours (Fig. 3). There was an increase to 80.00% (± 10.00% SEM) at 12 hours before reaching the highest percentage of infected fleas at 24-hours post-exposure to an infected bloodmeal

(85.00% ± 7.64% SEM). After removing the infected bloodmeal, the percentage of infected fleas decreased dramatically to 45.00% (± 2.24% SEM) at 48-hours. This downward trend continued, as percentage levels reached 31.67% (± 10.14% SEM) at 72-hours and remained relatively stable at 40.00% (± 4.47% SEM) after 96-hours. Infection prevalence was 25.63% (±8.32% SEM) for the final timepoint at 120-hours post infection. Thus, these data demonstrate that the cat flea gut is susceptible to *S. marcescens* infection, but this bacterium does not proliferate and is cleared following removal of an infected bloodmeal.

Suppression of ROS increased S. marcescens infection in the flea gut

After successfully establishing flea infections with *S. marcescens*, the role of ROS in the gut immune response of cat fleas was examined. Specifically, ROS levels were suppressed by feeding cat fleas an antioxidant (N-acetyl-L-cysteine) prior to infection. On average, antioxidant-treated fleas had higher bacterial loads (15,132 CFU ± 3637 SEM) compared to untreated fleas (4,583 CFU ± 720.50 SEM) (Figure 4; t-test: $p = 0.0016$). Overall, these data support the hypothesis that ROS limits survival of pathogens in the gut of actively feeding cat fleas.

ROS production is increased in response to S. marcescens infection

Given the higher bacterial loads in fleas fed an antioxidant, hydrogen peroxide (ROS) levels were measured in the four treatment groups (Table 1) to confirm ROS synthesis during an active infection. Of these treatment groups, *S. marcescens*-infected fleas had the highest levels of hydrogen peroxide compared to the control fleas (Fig. 5; Tukey's: $p < 0.005$). The *S. marcescens*-infected fleas' hydrogen peroxide levels were also significantly higher than the antioxidant + uninfected fleas and the antioxidant + infection fleas (Tukey's: $p < 0.0001$). Additionally, the antioxidant + uninfected group experienced a decrease of 75% in hydrogen peroxide levels compared to the control group (Tukey's: $p < 0.001$). Furthermore, the antioxidant + infection group (Group 4, Table 1) experienced a decrease of 70% and 50% as compared to the non-antioxidant treated groups (Groups 1 and 2, Table 1) (Fig. 5; Tukey's: $p <$

0.0001 and p = 0.0030, respectively). Taken together, these data show that ROS production increased in the presence of pathogenic bacteria compared to a bloodmeal alone and that antioxidant treatment reduces ROS prevalence within the flea gut.

Duox regulation is tightly controlled following infection by a bacterial pathogen

Given increased production of ROS in the flea gut, relative expression of genes associated with the DUOX expression pathway (*Duox*, *MEKK1*, *ATF2*, *MKP3*, *MEK3*, and *p38a*) were analyzed. Guts were dissected and pooled from the four previously used treatment groups (Table 1) at 24 hours. Additionally, an earlier time point was examined after fleas fed on an infected bloodmeal for four hours (Table 5). Of the six GOI, the four-hour infection group showed an increase in fold-change of 1.8 for mRNA levels of *p38a* compared to the control (Figure 6b; Tukey's: p = 0.0014). Both *MEKK1* and *ATF2* were downregulated in the infection groups at 24 hours and decreased in fold change by 0.41 and 0.45 for *MEKK1* (Tukey's: p = 0.0203 and p = 0.0129, respectively), and 0.46 and 0.48 for *ATF2* (Tukey's: p = 0.0212 and p = 0.0167, respectively) when compared to the control. Interestingly, no groups exhibited different levels of expression for *MKP3*, even though *ATF2* and *MEKK1* levels decreased at the 24-hour time point for both infection groups. Finally, there was no difference in *Duox* expression levels for any treatment group. Overall, these data provide evidence that the *Duox* expression pathway is tightly regulated in response to infection.

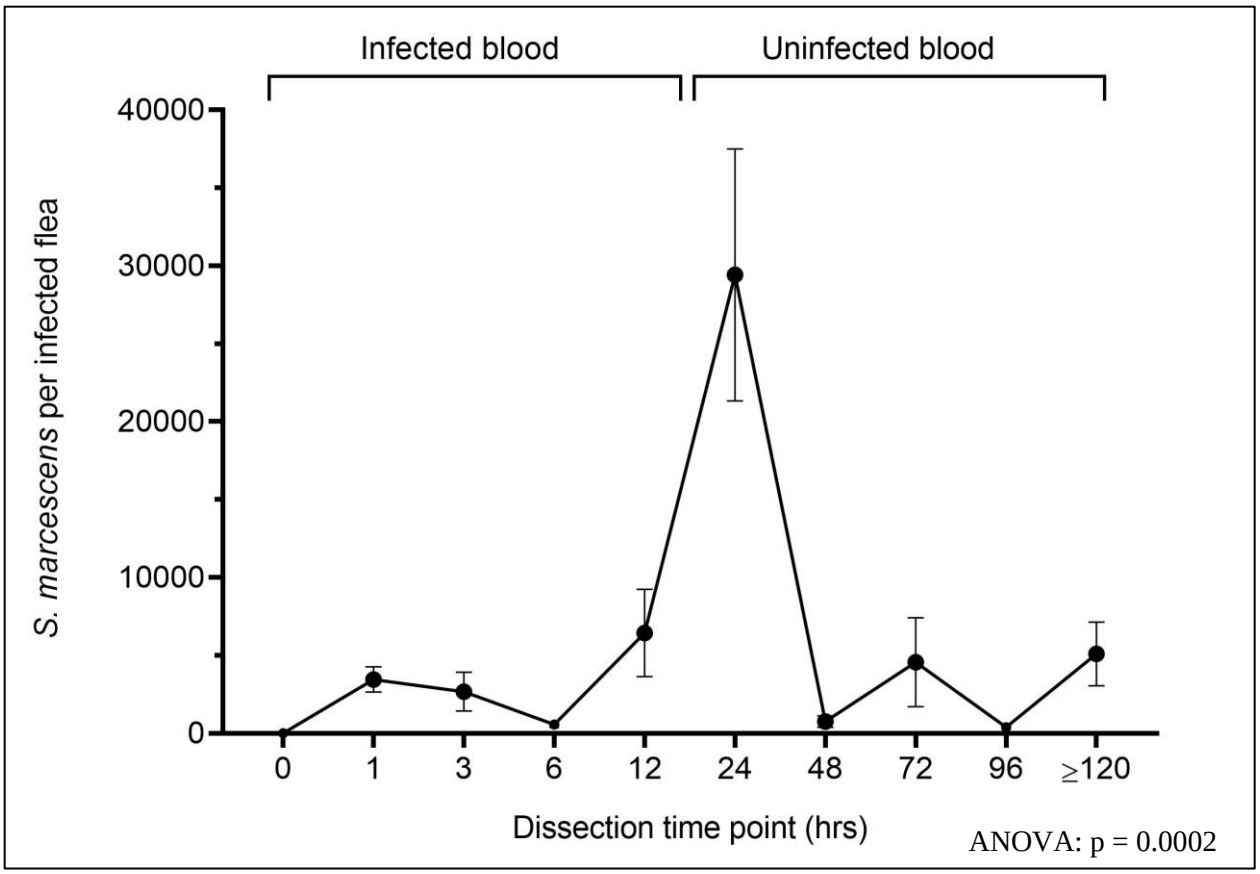

Figure 2. Timeline of infection intensity in cat fleas fed on *S. marcescens*-infected and uninfected blood. Circles mark the average CFUs, and error bars denote the standard error of the mean. Data were analyzed via ANOVA followed by Tukey's multiple comparisons test.

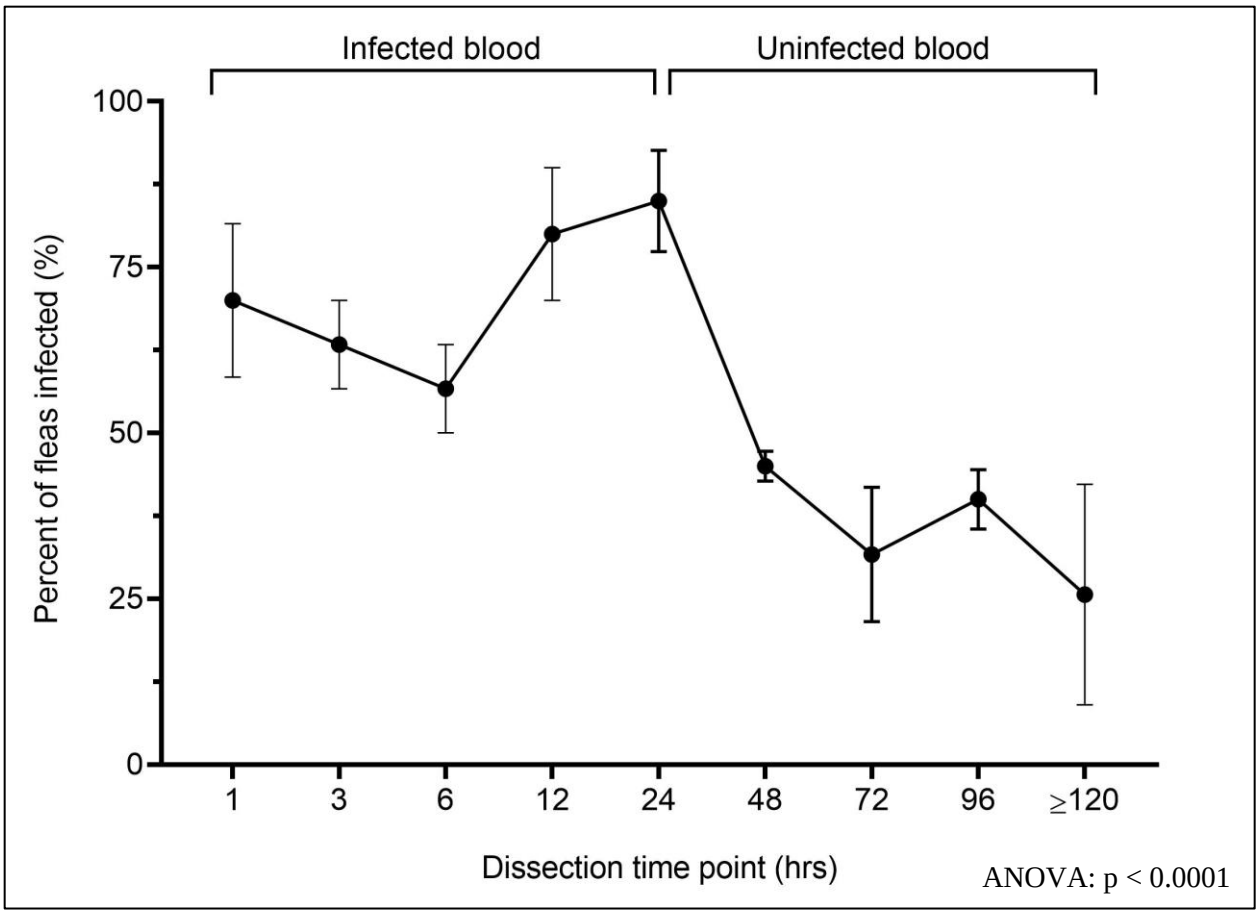

Figure 3. Timeline of infection prevalence in cat fleas fed on *S. marcescens*-infected and uninfected blood. Circles mark the average percent infected and error bars denote the standard error of the mean. Data were analyzed via ANOVA followed by Tukey's multiple comparisons test.

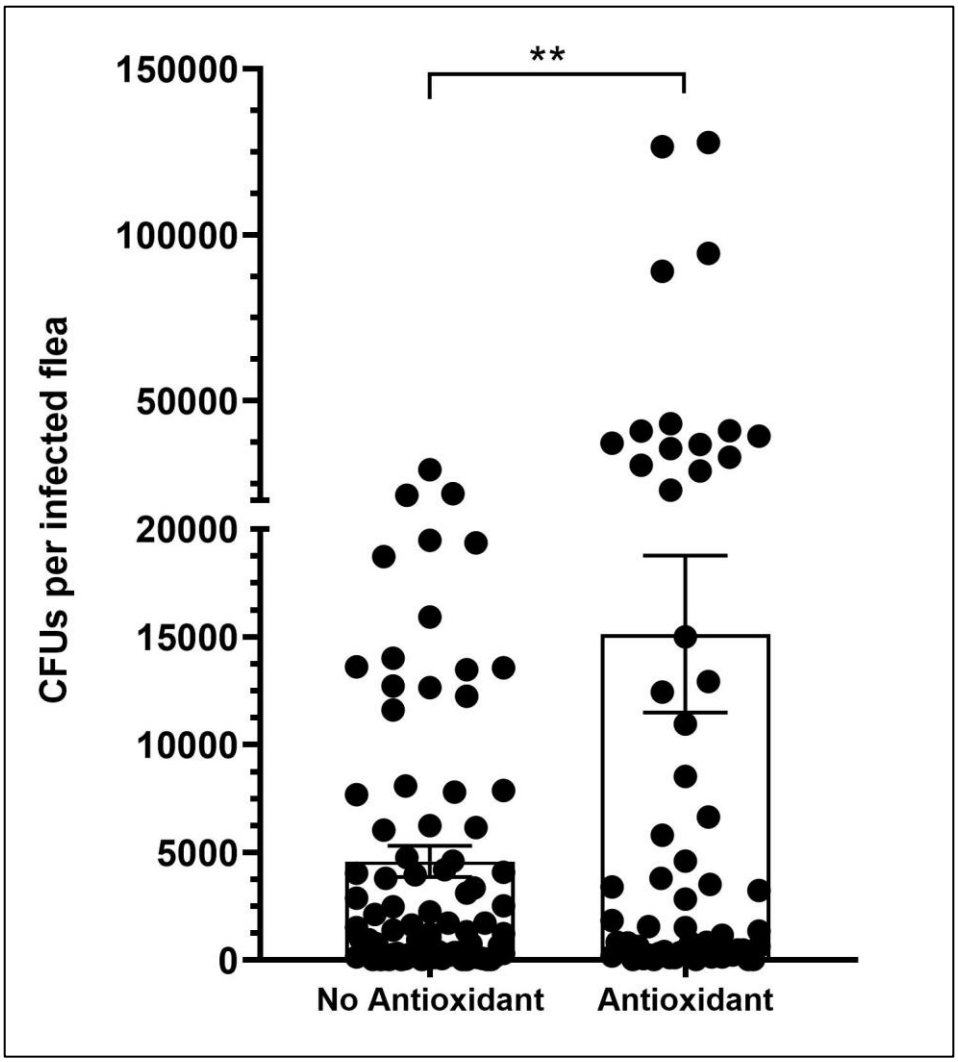

Figure 4. Intensity of *S. marcescens* infection in fleas administered an antioxidant prior to infection. Column heights represent the average infection intensity, error bars denote the standard error of the mean, and circles mark individual flea CFUs in each group. Data were analyzed via unpaired t-test. **$p < 0.005$.

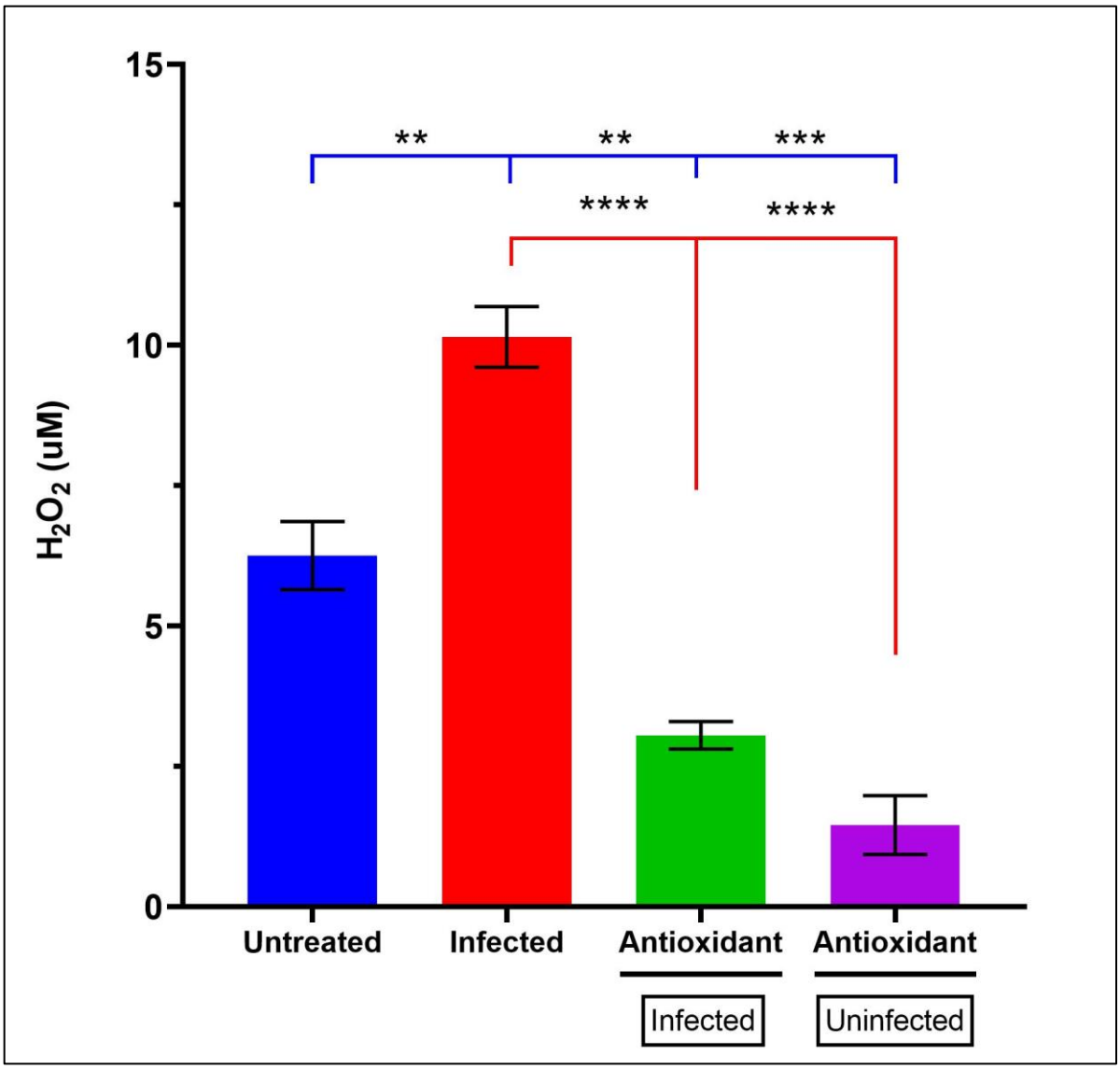

Figure 5. Hydrogen peroxide assay of *C. felis* after infection with *S. marcescens*. Column heights represent the average peroxide level and error bars denote the standard error of the mean. Data were analyzed via ANOVA followed by Tukey's multiple comparisons test. Blue lines represent comparisons between groups in relation to the untreated group, while red lines denote comparisons between groups in relation to the infected group. **$p < 0.005$; ***$p < 0.001$; ****$p < 0.0001$.

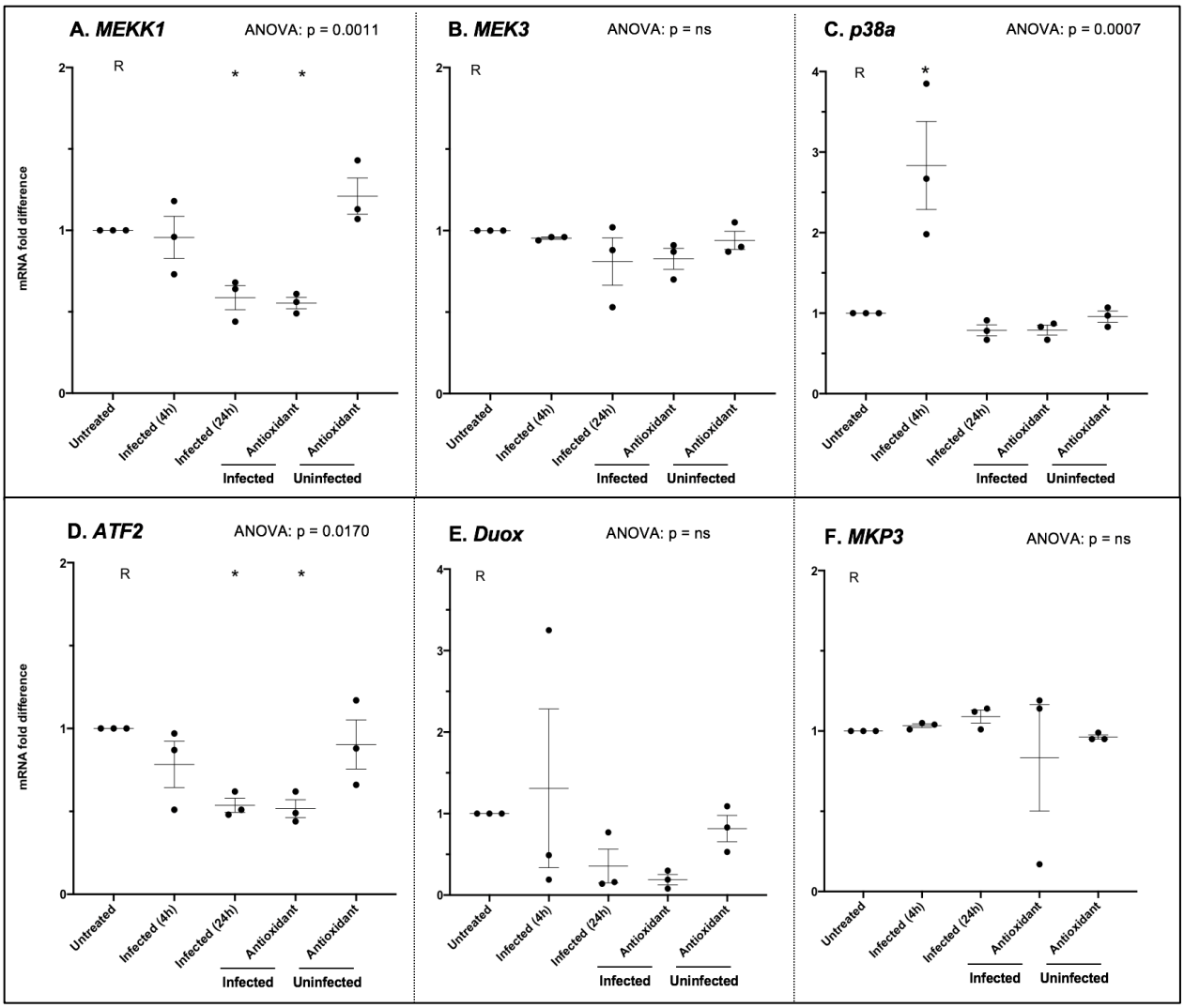

Figure 6. Relative quantification (RQ) of genes associated with the DUOX expression pathway: (A) *MEKK1*, (B) *MEK3*, (C) *p38a*, (D) *ATF2*, (E) *Duox*, and (F) *MKP3*. Data represent three independent trials. Circles mark the data points, horizontal line marks the mean, error bars denote the standard error of the mean. Data were analyzed via ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.005$.

CHAPTER 4

DISCUSSION

Despite their medical and veterinary importance, relatively few laboratories have studied the biology of fleas to the same extent as other disease vectors (mosquitoes, sandflies, ticks, and tsetse flies) (Brown 2019, Zug and Hammerstein 2015, Telleria *et al*. 2018). The ability of fleas to eliminate ingested pathogens ultimately determines their vectorial capacity (Brown 2019, Hinnebusch *et al.* 2021). The innate immune system found in insects, including fleas, generally targets foreign bodies, although some immune products are less specific and are capable of damaging host cells (Mikonranta *et al*. 2014). Flea-borne bacterial pathogens are typically confined to the flea gut during the early stages of infection (Bouhsira *et al*. 2013, Thepparit *et al*. 2013); thus, the surrounding epithelial cells must be armed with defense mechanisms to resist or limit infection (Legendre and Macaluso 2017, Lappin 2018). For example, the production of ROS in the gut of hematophagous arthropods is often the first line of defense against infected bloodmeals (Liu *et al*. 2012, Kim *et al*. 2017).

Two of the most important factors that influence the likelihood of a successful host defense include the mechanisms driving the immune response and how genes essential for immune function are regulated (Mikonranta *et al*. 2014). If the pathogen is successful in evading host defenses and persists within the flea, then downregulation of immune genes and ROS production will occur in order to limit damage to vital organs and tissues (Dreher-Lesnick *et al*. 2010). Additionally, the downregulation of immune genes could lead to proliferation of the pathogen, thus making them viable vectors for pathogen transmission. Furthermore, the intake of a bloodmeal has been implicated in decreasing ROS levels in other hematophagous insects (e.g., *Aedes aegypti*), thereby increasing their susceptibility to pathogens (Oliveira *et al*. 2011). Overall, the current study provides evidence of ROS production and differential gene regulation of the ROS-producing DUOX enzyme in response to infection by *S. marcescens* in the cat flea.

A major barrier to the study of host-pathogen interactions in fleas is the lack of a feasible infection model. Most flea-borne pathogens require a significant amount of maintenance (lengthy incubation periods), specialized equipment (biosafety cabinets and CO_2 incubators), and access to a high-containment biosafety level laboratory (BSL3 for work with the agents of plague and murine typhus). Among the large variety of commonly used bacteria, the selection of a model bacterium to use in this study must share similar properties to flea-borne pathogens. The use of *S. marcescens* as a bacterial pathogen was due to the following reasons: 1) it is a Gram-negative bacterium (a trait shared by *Rickettsia* and *Bartonella* spp.); 2) it is easily maintained in a BSL2 facility; 3) it forms distinct colonies to allow for easy calculation of bacterial concentration; 4) it has a distinct red pigmentation for easy differentiation from non-*S. marcescens* bacterial colonies; and 5) produces a significant amount of uracil, which is needed for activation of DUOX signaling pathways (Lee *et al*. 2013). Our study provides evidence that *S. marcescens* is an appropriate model pathogen based on the previously described criteria, and the subsequent immune response observed in the cat flea.

To establish an infection, it is often beneficial for the pathogen to avoid detection by the host. This can be accomplished in a variety of ways, with a common strategy being to invade the host cells (Campoy and Colombo 2009). The majority of fleas that fed on an *S. marcescen*-infected bloodmeal maintained infections for the entire exposure period before replacement with a non-infected bloodmeal. Infection intensity levels varied greatly across the designated time course; however, bacterial loads increased with exposure time to an infected bloodmeal. Moreover, both infection intensity and infection prevalence peaked at 24 hours, which was the last time point examined while fleas were still exposed to an infected bloodmeal. The decrease in infection intensity and infection prevalence following the replacement with an uninfected bloodmeal suggests that *S. marcescen* is ultimately eliminated as fleas actively feed and produce waste. This is similar to other flea-borne pathogens, such as *Y. pestis*, that cannot attach to or invade flea epithelial cells (Hinnebusch *et al*. 2021). Thus, the 24-hour time point was

considered optimal for detection of ROS production and upregulation of genes involved in the DUOX expression pathway.

Production of ROS by epithelial cells is a key regulator of microbes in the midgut for many different disease vectors (Taracena *et al*. 2018, Pan *et al*. 2012). The results of the antioxidant experiments and hydrogen peroxide assays confirmed our initial hypothesis that ROS production provides defense against ingested pathogens. These data indicate that the introduction of an antioxidant increased S. *marcescens* bacterial loads within the cat flea gut. Further, in infected fleas, peroxide levels were significantly elevated as compared to uninfected individuals, confirming its increase in production as a response to pathogenic bacteria. Additionally, fleas fed the antioxidant, N-acetyl-L-cysteine, experienced significantly decreased peroxide levels compared to both the infection and control groups. These higher pathogen loads indicate that ROS production is sufficient in regulating *S. marcescens* levels, and detoxification of ROS increases cat flea susceptibility to infection. These results are consistent with other studies investigating ROS production in other insects such as fruit flies, mosquitoes, and Oriental rat fleas (Ha *et al.* 2005a, Ha *et al.* 2009a, Kumar *et al*. 2010, Zhou *et al*. 2012). Taken together, the results identify ROS as an important early immune response in the cat flea gut.

Because free radicals damage host cells alongside foreign microbes, signaling pathways for DUOX-dependent ROS production are tightly regulated (Lambeth *et al*. 2007, Bae *et al*. 2010, Lee and Lee 2013). A dynamic cycle of gene expression is expected as continued production of DUOX proteins leads to extremely high levels of ROS (Ha *et al*. 2005b, Kim and Lee 2014). Given the increase of ROS levels in the flea gut, selected genes associated with the DUOX expression pathway were monitored in response to infection. Of the six genes examined, only expression of *p38a* was upregulated in response to infection by *S. marcescens*. This gene is known to be a vital kinase in the phosphorylation cascade needed for activation of *Duox* transcription (Kim and Lee 2014). However, *Duox* expression was unchanged at the time points examined, further implicating that expression of these genes are strictly regulated to protect host cells from damage by oxidative stress. Increased expression of *Duox* directly leads to

increased production of DUOX proteins; yet *Duox* expression levels in the *Drosophila* midgut are also found to be relatively modest (Ha *et al*. 2005b). Tight regulation of the DUOX expression pathway was corroborated by downregulation of *ATF2* and *MEKK1* at 24 hours, even though H_2O_2 levels were high within the flea gut at that time point. While these results were mixed, they provide an indication of the tight regulation of this pathway for ROS production. A recent study by Dahmani *et al*. (2020) found upregulation of genes associated with ROS production in tick cells infected by a rickettsial pathogen, *Anaplasma phagocytophilum*. Further, because our study was conducted *in vivo*, it provides a more accurate picture of the immune response in cat fleas following infection. For most studies, *in vitro* experiments investigating gene expression, while good predictors, tend to experience higher gene regulation (up or down), as opposed to *in vivo* (Dahmani *et al*. 2020). Identification of upregulation for even a single gene (*p38a*) gives further credence that the DUOX expression pathway is integral for ROS production in response to infection of cat fleas.

A possible explanation for the observed discrepancy may be due to a lag time between pathway gene expression and ROS levels, as a result of the increased production of DUOX proteins on the surface of host cells (Ha *et al*. 2005b). The inability to screen fleas prior to dissection could also confound gene expression analysis as noninfected fleas are likely included in the pool. In the future, labeling the bacteria with a fluorescent tag would alleviate the issue as flea guts could then be screened prior to pooling (Jakobs *et al*. 2000, Diaz-Albiter *et al*. 2011). Furthermore, research examining calcium levels in flea cells could help to identify the appropriate timeframe for dissection and analysis. Calcium is needed for DUOX activation, and changes in concentrations from the endoplasmic reticulum (the housing site of cellular calcium) would indicate the earliest timepoint for ROS production (Ha *et al*. 2009b, Kim and Lee 2014). Taken together with this study, these modifications could identify the entirety of the early flea gut immune response.

Conclusion

This study provides evidence that ROS is a key mechanism for early gut defense against pathogenic bacteria in the cat flea, and its production is firmly regulated to protect host cells. Further, *S. marcescens* is an appropriate model for the study of flea immunity because of its ease of use in the laboratory and its ability to elicit an immune response within cat fleas. Additionally, the increasing prevalence of *R. typhi* and *B. henselae* highlights the need to understand how fleas succumb, transmit, or eliminate infections (Bitam *et al*. 2010, Eisen and Gage 2012, Blanton *et al*. 2019) The relative capacity of fleas to eliminate an infection is also vital to determine their ability to spread flea-borne pathogens, as some individuals may be more susceptible to infection than others. Overall, these findings are critical to understanding cat flea immunity and their ability to transmit bacterial pathogens to humans and other hosts.

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